



## Review

Porcine circovirus: Transcription and DNA replication<sup>☆</sup>Andrew K. Cheung<sup>\*</sup>

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## ABSTRACT

This review summarizes the molecular studies pertaining to porcine circovirus (PCV) transcription and DNA replication. The genome of PCV is circular, single-stranded DNA and contains 1759–1768 nucleotides. Both the genome-strand (packaged in the virus particle) and the complementary-strand (synthesized in the new host) encode viral proteins. Among a multitude of RNAs synthesized by alternate splicing, only *rep* and *rep'* are essential for virus DNA replication via the rolling-circle replication (RCR) mechanism. In contrast to other RCR biological systems which utilize only one multi-functional protein, Rep, to replicate their respective genomes, PCV requires two proteins, Rep and Rep'. During DNA synthesis, the PCV origin of DNA replication (Ori), which contains a pair of inverted repeats (palindrome), exists in a destabilized four-stranded configuration (the melting-pot model) and permits both the palindromic-strand and the complementary-strand to serve as templates simultaneously for initiation and termination. Inherent in the "melting-pot" model is the template-strand-switching mechanism. This mechanism is the basis for the "correction or conversion" of any mutated nucleotide sequences engineered into either arm of the palindrome and the incorporation of "illegitimate recombination" (addition or deletion of nucleotides) events that are commonly observed at the Ori of other RCR biological systems during DNA replication.

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## 1. Introduction

The *Circoviridae* family consists of a group of diverse non-enveloped animal viruses that have small, monomeric, single-stranded (ss), closed-circular DNA genomes which are replicated via doubled-stranded intermediates (Fauquet and Fargette, 2005). There are two genera in the *Circoviridae* family. Members of the genus *Circovirus* that infect birds and pigs have ambisense genomes and both the virion and complementary strands code for viral proteins. Members of the genus *Gyrovirus*, chicken anemia virus and a newly reported human gyrovirus (Sauvage et al., 2011), have negative-sense genomes and only the complementary plus-strand codes for viral proteins. Phylogenetically, members of the *Circoviridae* family are related to viruses from the plant families *Geminiviridae* and *Nanoviridae* (Niagro et al., 1998). Sequence analysis of the replication initiation protein, Rep, suggested that animal circoviruses were derived from a plant nanovirus that switched hosts via an insect vector to infect a vertebrate and then recombined with a ss-RNA vertebrate-infecting virus (Gibbs and Weiller, 1999). Recently, a new genus of viruses that has an ambisense genome and infects humans and wild chimpanzees, *Cyclovirus*, has been proposed as a new member of the *Circoviridae* family (Blinkova et al., 2010; Li et al., 2010).

## 2. Porcine circovirus

### 2.1. Genome organization

Porcine circovirus type 1 (PCV1) is the type specific virus of the *Circovirus* genus. The virion is non-enveloped, icosahedral, 16–21 nm in diameter and contains a closed-circular, ss-DNA genome of 1759 nucleotides (nt), which makes it one of the smallest known animal viruses. PCV1 was discovered in 1974 as a contaminant of a porcine kidney (PK15) cell line. While PCV1 is non-pathogenic (Tischer et al., 1986, 1974), a second genotype of porcine circovirus (PCV2 of 1768 nt) is associated with post-weaning multi-systemic wasting syndrome in swine (Clark, 1996; Harding, 1996).

The genome organization of PCV1 and PCV2 is very similar. The *cis*-acting and *trans*-acting replication factors of both viruses are interchangeable for DNA replication and for chimeric virus production (Beach et al., 2010a,b; Cheung, 2003b; Fenaux et al., 2004; Mankertz et al., 2003). Typically, the origin of DNA replication (Ori) is represented by a “stem-loop” structure associated with several notable *cis*-acting elements (Fig. 1a). The stem is formed by base-pairing of 11-base pair (bp) inverted repeat sequences (palindrome), while the loop contains 10–12 nt (12 for PCV1 and 10 for PCV2) which include a conserved octanucleotide motif sequence ( $A_1x_2T_3A_4x_5T_6\downarrow A_7C_8$ ) (Oc8) ( $\downarrow$  indicates the nick or cleavage site). A six bp tandem direct repeat sequence (designated H1/H2) is present abutting the right-arm of the stem-loop structure. There are two open reading frames, ORF1 and ORF2, which diverge from the Ori (Fig. 2). ORF1 is a clockwise virion-strand *rep*-gene region that encodes two replication proteins (Rep and Rep') and ORF2 is a complementary-strand *cap*-gene region that encodes the capsid protein (Cap). The 5'-ends of these two ORFs are separated by the Ori-intergenic region (IR) and the 3'-ends are separated by the termination-IR.

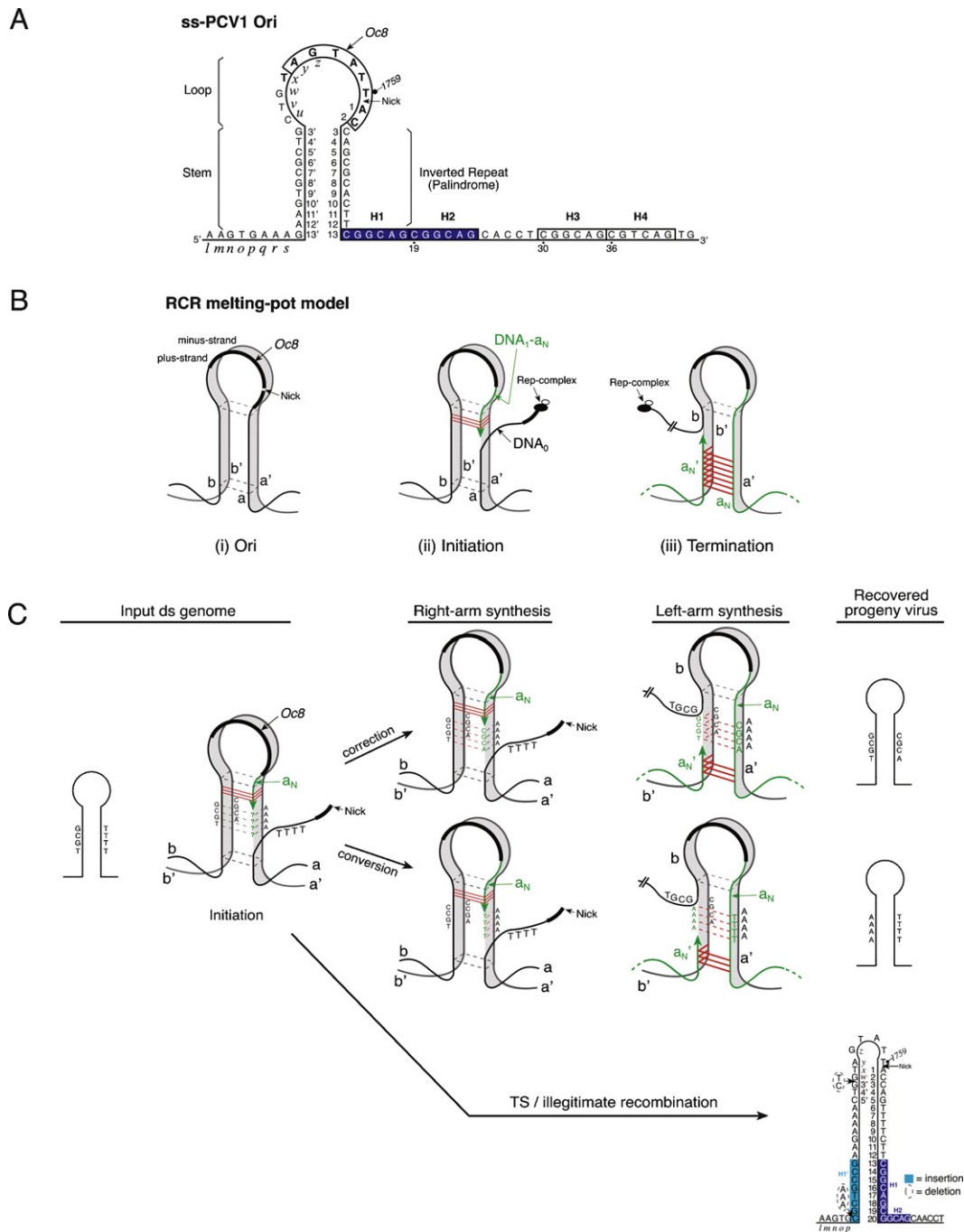
### 2.2. Transcription

PCV exhibits a very complex transcription pattern. RNA synthesis is carried out by cellular enzymes after the ss viral genome is converted to a ds molecule in the new host. The transcription strategy is bidirectional and various viral RNAs are produced via alternate splicing. PCV1 and PCV2 utilize similar initiation and termination signals at comparable locations within the viral genome; however, they differ from each other with respect to specific RNA expression level as well as splice-junction selection unique to each virus (Cheung, 2003a,c). Thirteen RNAs have been reported for PCV1 and ten for PCV2 during virus replication in PK15 cells (Fig. 2) (Bratanich and Blanchetot, 2002; Chaikyakul et al., 2010; Cheung, 2003a,c; Liu et al., 2005). The RNA profile of PCV2 is presented to illustrate the complexity and potential coding capacity of the virus. The complementary-strand codes for two transcripts, *cap* which encodes the viral capsid protein (Cap) and *ORF3-RNA* which encodes the apoptosis-associated protein. While the function of the respective protein is the same, ORF3 of PCV1 is twice the size of PCV2 (Chaikyakul et al., 2010). The promoter for PCV1 *cap*, but not for the *ORF3-RNA*, has been mapped (Mankertz and Hillenbrand, 2002). *Cis*-acting elements (Sp1, TATA, ISRE and USF/MLTF), commonly associated with eukaryotic promoters, are present in the *cap*-promoter. From the *rep*-gene region, a cluster of five *rep*-associated RNAs (*Rep*, *Rep'*, *Rep3a*, *Rep3b* and *Rep3c*) and three minor *NS*-associated RNAs (*NS1515*, *NS672* and *NS0*) have been detected. The *rep*-associated RNAs all share common 5' and 3' nucleotide sequences, which indicate that they are, likely, derived from the full-length *rep* via alternate splicing. The promoter for PCV1 *rep*, but not for the minor RNAs, has also been mapped (Mankertz and Hillenbrand, 2002). Eukaryotic *cis*-acting elements (AP3, Sp1 and AP2), are observed in the *rep*-promoter. The minor *NS*-associated RNAs that share 3' common nucleotide sequences with the *rep*-associated RNAs are likely synthesized from three different promoters. Mutations (start codon modification, in frame termination and splice-junction alteration) introduced into these RNAs showed that only *Rep* and *Rep'* are essential for viral DNA replication (Cheung, 2003b, 2004c; Liu et al., 2005).

The polypeptides encoded by *rep*, *rep'*, *cap* and *ORF3-RNA*, have been characterized. However, whether the minor RNAs encode proteins or what functions each plays in the life cycle of PCV is not clear. Only *Rep* and *Rep'* are essential for viral DNA replication in mammalian cells (Cheung, 2006; Mankertz and Hillenbrand, 2001). The *ORF3*-associated proteins of PCV1 and PCV2 induce apoptotic cell death (Chaikyakul et al., 2010; Liu et al., 2005). The capsid protein encapsulates the ss circular viral genome to form infectious virions and it may play a role in transporting the *Rep* and *Rep'* proteins from the cytoplasm into the nucleus to facilitate PCV DNA replication (Cheung and Greenlee, 2011; Lin et al., 2009).

### 2.3. The *Rep*-complex

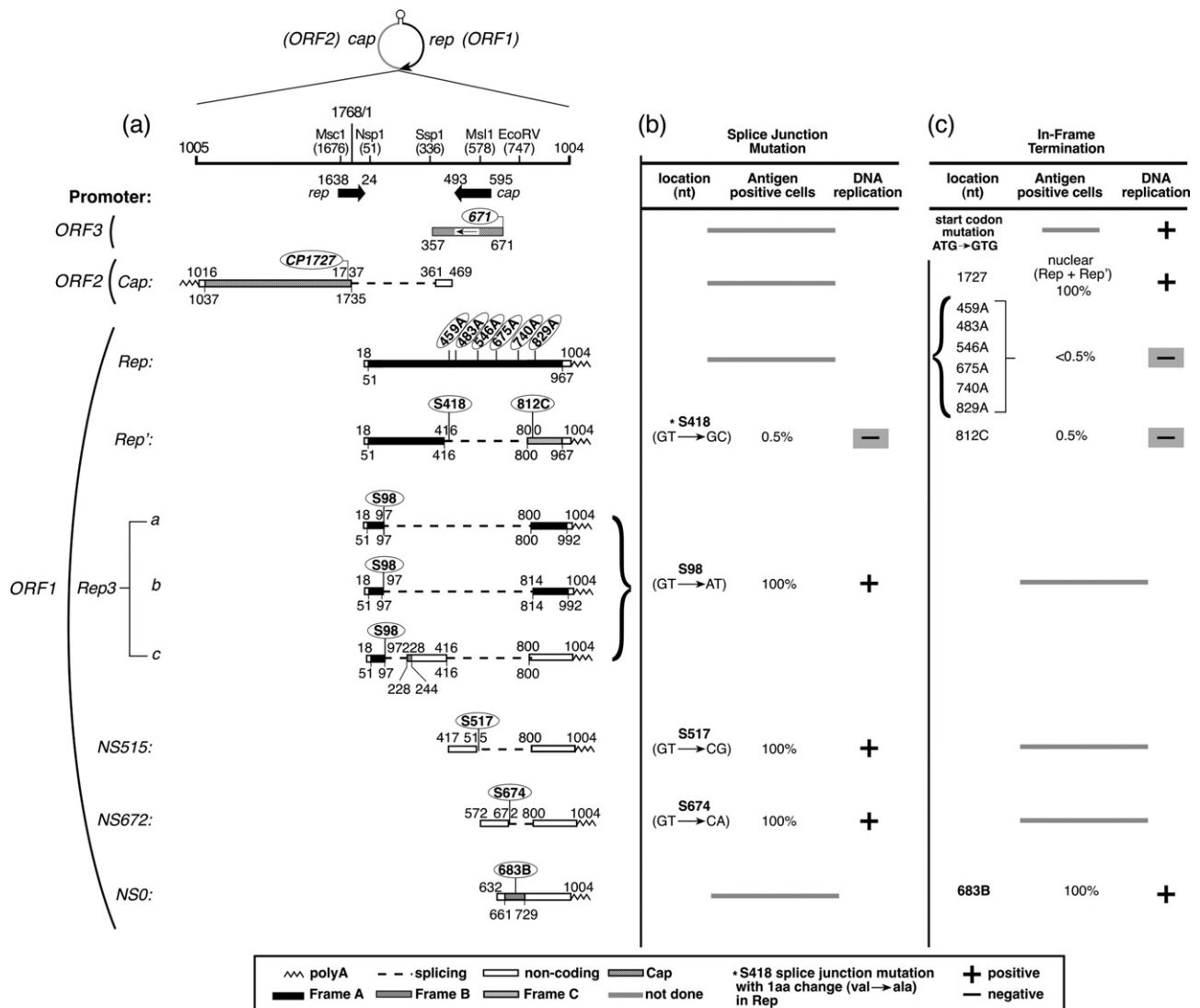
The ORF1 *rep*-gene region codes for two replication initiation proteins, *Rep* and *Rep'*. *Rep* (312 amino acid residues) is encoded by the entire ORF1. It contains the signature amino acid motifs commonly associated with RCR found in the *Rep*-protein of other prokaryotic and eukaryotic systems (del Solar et al., 1993; Gutierrez, 1999; Ilyina and Koonin, 1992; Mankertz et al., 1998). These conserved motifs include RC-I (unknown function), RC-II



**Fig. 1.** (a) Schematic representation of the PCV1 Ori indicating potential base-pairing of the flanking inverted repeats. The genome sequence (1759 nt) and co-ordinates (1, 2, 3, ...) are based on GenBank accession number AY184287. The nt co-ordinates (3', 4', 5', ...) are arbitrarily assigned to show nt complementarity of the palindromic sequences. The Oc8 containing the nick site (AGTATTAC) is boxed and indicated in bold letters. The 6-nt tandem repeats (H1 to H4) located at nt 13, 19, 30 and 36 (not perfect at nt 38) are in boxes. H1 and H2 are indicated in blue. Relevant nt sequences are assigned arbitrary positions (l-m-n-o-p-q-r-s and u-v-w-x-y-z) to assist in retracing the templates used during replication (see c). (b) The RCR "melting-pot" model. A four-stranded structure that provides two templates, simultaneously, for DNA synthesis and permits TS during initiation and termination of DNA replication. The minus-genome is indicated in gray, the parent genome (DNA<sub>0</sub>) is in black and the first round genome (DNA<sub>1</sub>) is in green. The nascent right-arm palindrome strand (at initiation) is designated a<sub>N</sub> and its complementary strand (at termination) is designated a'<sub>N</sub>. Red lines indicate potential base-pairing opportunities with two templates. (c) Schematic diagram showing the generation of progeny viruses with mutated palindromic sequence via the TS mechanism. The mutated input genome may revert to a wild-type palindrome (correction), adopt a new palindrome (conversion) or incorporate nucleotide deletion/insertion (illegitimate recombination) to yield viable viruses.

(divalent ion coordination), RC-III (contains a tyrosine residue for introducing a nick at the Oc8 sequence of the Ori and covalent linkage to the 5'-end of the displaced DNA), and P-loop (a putative helicase domain that exhibits ATPase activity in the presence of divalent ions) (Ilyina and Koonin, 1992; Steinfeldt et al., 2007). Mutational studies showed that RC-I, RC-II, and RC-III are essential for Ori cleavage and PCV DNA replication in cell culture, while the

P-loop is non-essential for Ori cleavage *in vitro* but indispensable for DNA replication in cell culture (Steinfeldt et al., 2007). Rep' (168 amino acid residues) is translated from an internally spliced RNA of ORF1. Rep and Rep' are identical at the N-terminal portion, but differ at the C-terminal portion because Rep' is spliced at the 3' portion to a different ORF. Rep' contains RC-I, RC-II, and RC-III, but not the P-loop.



**Fig. 2.** The essential and non-essential RNAs of PCV2. (a) Promoters and transcripts. A schematic representation of the PCV2 genome, the promoters for *rep* and *cap* and the RNAs encoded by ORF3 (with orientation indicated by an arrow), ORF2 and ORF1 are indicated. The nucleotide sequence is based on GenBank accession number AY094619. The RNAs are annotated with nt coordinates that indicate the last nucleotide of each respective exon. The coding sequence of each RNA was shaded and their nt coordinates are indicated below each RNA. Mutations introduced into the RNA are indicated in bold and enclosed in oval boxes. (b) Summary of splice junction mutations. (c) Summary of in-frame mutations.

The fact that Rep and Rep' contain identical RCR motifs at the 5' portion but differ at the 3' portion, it is expected that these two proteins can exhibit similar activities, but not all, under the same experimental conditions. Both Rep and Rep' co-localize to the nucleus but bind the ds-PCV Ori DNA sequences with slightly different specificity (Finsterbusch et al., 2005; Steinfeldt et al., 2001). Rep binds to the right-arm of the palindrome and the direct repeat H1/H2 sequences, while Rep' only binds the H1/H2 sequences. Both Rep and Rep' exhibit the nicking/joining activity of the ss Oc8 sequence under identical experimental conditions *in vitro*. The nicking activities of Rep and Rep' are dependent on the presence of divalent ions, but not the presence of ATP. However, the cleavage activity of Rep, but not Rep', is stimulated by ATP. Both Rep and Rep' can covalently link to the 5'-end of the nicked DNA after cleavage (Steinfeldt et al., 2007) and possess a nucleotidyltransferase activity that can religate ss Oc8 together (provided that the protein is covalently linked to the 5'-end of the cleaved Oc8) only in the presence of a ss stem-loop (sequence non-specific) structure. Apart from the helicase activity predicted for Rep (P-loop), it has

been demonstrated that Rep, but not Rep', can modulate *rep* transcription by repressing the *rep* promoter via RC-II and the P-loop (Mankertz and Hillenbrand, 2002).

Rep and Rep' can form homo-complexes (Rep–Rep and Rep'–Rep') and hetero-complexes (Rep–Rep') (Mankertz and Hillenbrand, 2002). Since Rep and Rep' are both required for PCV DNA replication, the hetero-complex Rep–Rep' is generally referred to as the Rep-complex. The fact that the ratio of Rep versus Rep' RNAs changes during the course of a productive infection suggests that a different complex may be predominant at different times during DNA replication and that each type of complex may play a unique role in specific reactions. Although other RCR biological systems require only a Rep protein to replicate their DNA genomes, PCV needs both Rep and Rep'. It is interesting to note that the excision of a unit length ds viral genome from a bacterial plasmid containing head-to-tail tandem PCV genomes which requires the nicking of Oc8 during initiation and reconstituting Oc8 during termination, can be carried out solely by Rep in *Escherichia coli* (Cheung, 2006).



### 3. DNA replication

PCV replicates its genome via RCR, a mechanism that is widely used in bacterial plasmids, phages, and plant as well as animal viruses (del Solar et al., 1998; Graham et al., 1989; Hanley-Bowdoin et al., 2000; Khan, 2000; Musatov et al., 2000; Novick, 1998) and the replication process is carried out by cellular enzymes expressed during S-phase of the host cell cycle (Tischer et al., 1987). Upon infection, the virion uncoats and the ss viral genome is transported to the nucleus. A model for PCV genome replication is outlined in Fig. 3. The initial parent viral genome, the first round progeny genome and the second round progeny genome are designated DNA<sub>0</sub> (in black), DNA<sub>1</sub> (in green) and DNA<sub>2</sub> (in orange), respectively. The minus-strand genome is in gray.

#### 3.1. Step 1: Genome conversion from ss to ds

The ss DNA<sub>0</sub> viral genome is converted to a ds-intermediate (ds-RF) which then becomes a superhelical molecule and serves as the template for viral DNA synthesis. The mechanism may be similar in bacterial plasmids, in which it has been shown that only supercoiled molecules are used as substrate for RCR initiation (Khan et al., 1981). The ss to ds genome conversion requires a minus-strand primer to initiate nascent complementary DNA synthesis. In geminiviruses and nanoviruses, the minus-genome initiation primer is composed of DNA or DNA with several 5' ribonucleotides packaged into the virion (Donson et al., 1984; Hafner et al., 1997) or, alternatively, by a RNA primer synthesized in the host after infection (Saunders et al., 1992). These primers are located in the initiation-IR or in the termination-IR. A similar mechanism may occur in PCV; however, the minus-genome primer for PCV has not been determined.

Double-stranded inverted repeats of 8, 11 and 17bp have been shown to give rise to four-stranded DNA structures by electron and atomic force microscopy (Kato et al., 2003, 1998; Ohta et al., 1996). In these studies, cruciform structures were never observed. Together with enzymatic and chemical analyses, a quadruplet model consisting of two ss stem-loops (hairpin structures) arranged in parallel and allowing all four strands to interact with each other was proposed. One stem-loop melts and donates a strand (the third strand) to the other stem-loop forming a triple-stranded stem with a ss extension (hairpin-triplex). Whether the 11-bp palindrome at the Ori of PCV exhibits this four-stranded structure naturally is not known.

#### 3.2. Step 2: Initiation of DNA replication

PCV genome replication requires the interactions of the Rep-complex (Rep–Rep') and the *cis*-acting element (in the appropriate conformation) at the Ori present in the minimum-binding-site recognition region (111 nucleotide for PCV1) of the viral genome (Mankertz et al., 1997). To initiate plus-sense genome DNA<sub>1</sub> synthesis, the Rep-complex binds the ds-RF hexanucleotide H1/H2 direct repeats (Steinfeldt et al., 2007) abutting the right-arm of the palindrome. This interaction induces conformational changes that include the ds Oc8 motif located in the loop to unwind and exhibit ss characteristics for the Rep-complex to nick the ss DNA<sub>0</sub> Oc8 (A<sub>1</sub>x<sub>2</sub>T<sub>3</sub>A<sub>4</sub>x<sub>5</sub>T<sub>6</sub>↓A<sub>7</sub>C<sub>8</sub>) sequence between T<sub>6</sub> and A<sub>7</sub> (Steinfeldt et al., 2006). The positions with specified nucleotides are essential for DNA replication, while positions indicated by x can accept variable nucleotides to yield progeny viruses (Cheung, 2004a). In geminiviruses, strain-specificity is conferred by the N-terminal portion of the Rep protein and the direct repeats present at the Ori (Choi and Stenger, 1995, 1996; Jupin et al., 1995). It is generally accepted that Rep, but not Rep', plays a role in the unwinding of the ds Oc8 sequence since it contains a helicase P-loop domain.

Cleavage of the phosphodiester bond between T<sub>6</sub> and A<sub>7</sub> of DNA<sub>0</sub> is catalyzed via nucleophilic attack by the hydroxyl group (–OH) of tyrosine 93 (Tyr<sub>93</sub>) present in motif RC-III of the Rep protein (a reaction tentatively assigned to Rep but not Rep') (Steinfeldt et al., 2007). The nicking activity is dependent on divalent cations (function of RC-II), but independent of ATP hydrolysis (function of P-loop) or presence of the palindromic sequences (stem-loop structure). After cleavage, Rep is covalently linked to the displaced 5'-end of DNA<sub>0</sub> via a Rep-tyrosinephosphodiester bond and generates a free 3'-OH end (DNA<sub>0</sub>-T<sub>6</sub>-OH) that serves as a primer for nascent DNA<sub>1</sub> unidirectional DNA synthesis. *In vitro* studies have shown that both Rep and Rep' are capable of nicking the ss Oc8 and covalent linkage to the displaced DNA<sub>0</sub> through the tyrosine residue (Steinfeldt et al., 2006). It is clear that the molecule that mediates the nicking is the one that forms the covalent linkage. However, direct evidence specifying whether Rep or Rep' mediates the nicking and attachment activity at initiation of PCV DNA replication has not been reported.

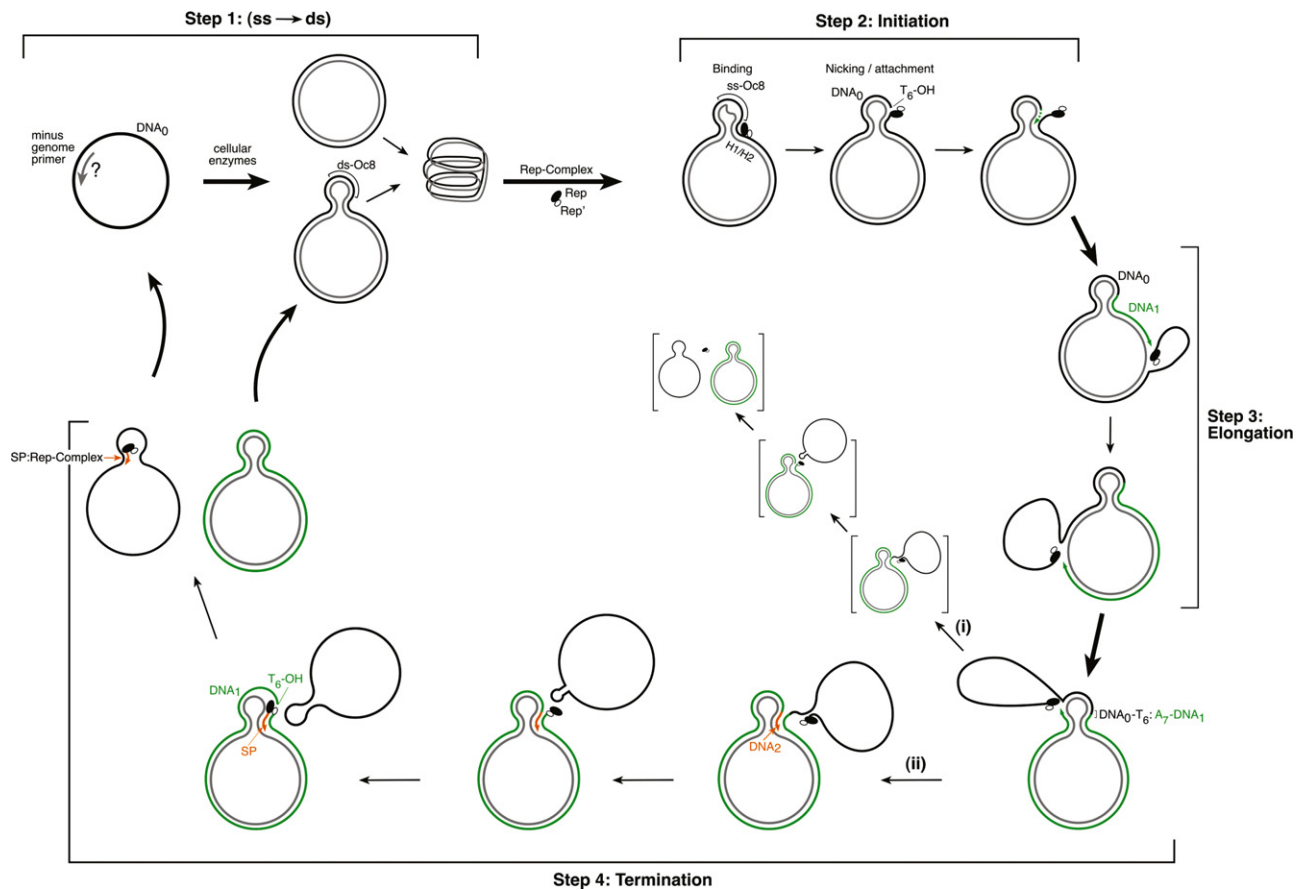
When the Rep-complex binds the ds H1/H2 direct repeats, it introduces a sphere of instability with conformational changes that includes the 10–12 bp loop and the 11 bp palindrome. The loop exhibits ss characteristics for Oc8 cleavage and the ds stem is induced to form a four-stranded structure (the “melting-pot” model) (Fig. 1b) (Cheung, 2004a) similar to the hairpin-triplex. Although the presence of the stem-loop configuration is not essential for Oc8 cleavage (Cheung, 2007; Steinfeldt et al., 2006), the “melting-pot” is predicted to be in existence during synthesis of the palindromic sequences at initiation and termination of PCV DNA replication. In the “melting-pot” model, the inverted repeats (strand-a, -a', -b and -b') are in a “melted” state with weakened or broken hydrogen-bonding among the palindromic sequences of the minus- and plus-strand genome of the ds-RF. The DNA strands remain in close proximity to each other and are juxtaposed in a four-stranded tertiary structure. Both the complementary a'-strand and the palindromic b-strand are available as templates, which allows template-strand-switching (TS), for synthesis of the nascent DNA<sub>1</sub> strand-a<sub>N</sub> while displacing the old viral DNA<sub>0</sub> genome with the Rep-complex covalently attached.

#### 3.3. Step 3: Elongation

The newly generated 3'-OH end (DNA<sub>0</sub>-T<sub>6</sub>-OH of the nicked Oc8), then serves as primer for first round DNA<sub>1</sub> synthesis. As neither Rep nor Rep' possesses any polymerase activity, elongation and synthesis of the nascent DNA<sub>1</sub> is expected to be performed by cellular enzymes. If the supercoiled configuration is essential for initiation of PCV DNA replication, the replicating genome will be unable to initiate another round of DNA replication until the first round of genome synthesis is completed.

#### 3.4. Step 4: Termination of DNA replication

Once the four-stranded hairpin-triplex configuration is induced by the Rep-complex during initiation, it is expected to remain in place. As DNA<sub>1</sub> synthesis reaches the left-arm of the palindrome, the “melting-pot” is in existence to provide two templates (strand-a<sub>N</sub> and strand-b') for synthesis of the left-arm strand-a'<sub>N</sub> palindromic sequence (Fig. 1b). Provided that palindromic base-pairing occurs to re-establish a stem-loop structure, reconstitution of Oc8 and termination of DNA replication will commence to yield a ss closed circular DNA<sub>0</sub> genome (Cheung, 2007; Steinfeldt et al., 2006) when synthesis of the entire new DNA<sub>1</sub> and displacement of the old DNA<sub>0</sub> are completed.



**Fig. 3.** A replication model for PCV. The parent virion-strand genome ( $\text{DNA}_0$ ) is represented by a black circle and the complementary genome strand synthesized in the new host is represented by gray circle. The first round DNA ( $\text{DNA}_1$ ) is indicated in green and the second round DNA ( $\text{DNA}_2$ ) is indicated in orange. SP:Rep-complex denotes the potential minus-genome primer molecule containing a short 10–12 nt of the Ori palindromic (SP) sequence covalently attached to the Rep-complex (see text for details).

At present, the precise termination event for PCV is not known. There are two potential scenarios. In both scenarios, Rep was assigned the nicking and attachment activities at initiation.

- (i) At the point when  $\text{DNA}_1$  is completely synthesized and  $\text{DNA}_0$  totally displaced, Rep' of the Rep-complex cleaves Oc8 between  $\text{DNA}_0\text{-T}_6$  and  $\text{A}_7\text{-DNA}_1$  (Fig. 3i). Rep' covalently attaches itself to the 5'-end of  $\text{DNA}_1$  and generates a free 3'-OH ( $\text{DNA}_0\text{-T}_6\text{-OH}$ ) end. Concomitantly, nucleophilic attack by this just freed  $\text{DNA}_0\text{-T}_6\text{-OH}$  on Tyr<sub>93</sub> of Rep-DNA<sub>0</sub> releases the displaced circular ss  $\text{DNA}_0$  and the Rep-complex is transferred to the 5'-end of  $\text{DNA}_1$  via Rep' covalent linkage. Nucleophilic attack mediated by the 3'-OH of  $\text{DNA}_1\text{-T}_6\text{-OH}$  on the newly transferred Rep'-Tyr<sub>93</sub>-DNA<sub>1</sub> reconstitutes the  $\text{DNA}_1$  Oc8 and releases the Rep-complex and a  $\text{DNA}_1$ -containing ds-RF intermediate. With this scenario, in the event an incompatible palindrome (naturally occurring or engineered) is present, this ds-RF molecule will be permanently disabled with a covalently linked Rep-complex.
- (ii) If DNA synthesis continues from first round  $\text{DNA}_1$  to second round  $\text{DNA}_2$  beyond the nick-site for a short distance (10–12 nt), a process similar to plasmid pT181 (Rasooly and Novick, 1993), the Rep-complex would undergo two separate nicking/joining events (first through Rep' and then through Rep) and result in a displaced ss  $\text{DNA}_0$ , a  $\text{DNA}_1$ -containing ds-RF intermediate and a Rep-complex with a short 10–12 nt palindromic sequence (SP) attached to Rep (SP:Rep-complex) (Fig. 3ii). Since Rep and Rep' both exhibit nicking activity in the absence of a palindrome (Steinfeldt et al., 2006), in the case of a mis-matched inverted repeat, nicking activity (possibly from a second Rep-complex) will continue without

formation of a closed circular ss  $\text{DNA}_0$  genome (but a linear  $\text{DNA}_0$  molecule covalently attached to the Rep-complex is released) and this  $\text{DNA}_1$ -containing ds-RF molecule will remain functional. If compatible palindromic sequences are restored in  $\text{DNA}_1$  (by correction or conversion), this ds-RF genome will then be able to yield viable genomes in subsequent rounds of DNA replication (Fig. 1c). This scenario can adequately account for the generation of viable progeny viruses from ds plasmid-derived PCV genomes containing incompatible inverted repeats (Cheung, 2004a,d).

#### 4. The “melting-pot” model

Derivation of the “melting-pot” RCR model was based on a series of mutagenesis studies at the PCV Ori (Fig. 1). This model provides the flexibility for the generation of a multitude of infectious viruses using ds PCV genomes derived from bacterial plasmids containing a variety of mutations engineered at the Ori (Fig. 1c). The recovered progeny viruses may contain different lengths and compositions of the loop, the palindrome and the H sequences. The “melting” state of the four-stranded inverted repeats provides two templates for palindromic sequence synthesis, simultaneously, during initiation and termination of PCV DNA replication (Cheung, 2004a). Inherent in the “melting-pot” is the TS mechanism that accounts for inverted repeat correction/conversion and the “illegitimate recombination” events (Michel and Ehrlich, 1986a,b) that commonly occur at the Ori during the RCR process. The H sequences also exhibit flexibility that affects the dynamics and outcomes at the Ori. There are 4 H sequences arranged in two tandems, the proximal H1/H2 and distal

H3/H4, to the right of the Ori inverted repeats (Fig. 1a). *In vitro*, there is a strict requirement that H1/H2 must abut the stem-loop structure for the Rep-complex to exert its biochemical capabilities (Steinfeldt et al., 2007). However, *in vivo*, mutations engineered into H3/H4 are retained in the progeny viruses, while mutations engineered into the H1/H2 are deleted and H3/H4 are placed next to the palindrome (Cheung, 2005b). If mutations are engineered into both H1/H2 and H3/H4, the genome may undergo “melting-pot” TS rearrangements and the recovered progeny viruses usually contain various combinations of H and H-like sequences.

However, the TS mechanism cannot explain the reversion of the nucleotides at the Oc8 critical position to wild-type nt without a template (Cheung, 2004b, 2005a). There are 12 nt in the PCV1 loop. Mutations engineered at the four 5' nt as well as the x-nt of Oc8 (O<sub>2</sub> and O<sub>5</sub>) are retained in the progeny viruses and mutations engineered into positions at O<sub>6</sub>–O<sub>8</sub> abolish DNA replication. Interestingly, mutations introduced into positions O<sub>1</sub>, O<sub>3</sub> and O<sub>4</sub> are able to revert back to wild-type nucleotides without a complementary DNA template. Whether this is a random incorporation process or the Rep-complex plays a role in determining the nucleotide at that specific site is not clear. There may be other forces at work at the Ori that are not recognized at this time or the cellular environment provides the flexibility and plasticity that determines the outcomes of the RCR process.

## 5. Concluding remarks

In this review, a summary of recent advances in elucidating the molecular events in the PCV DNA replication process were presented. The ability of the PCV Ori to accommodate a variety of modifications and yet maintain the basic core architecture is remarkable. The environment provided by the “melting-pot” is so flexible that mutations engineered into the inverted repeats or the H1/H2 sequences can be deleted, corrected or regenerated by orchestrating through the TS mechanism. It is likely that the “melting-pot” model is applicable to other biological systems that utilize RCR.

Much progress has been made in identifying the *cis*-acting elements and delineating the biochemical capabilities exhibited by Rep and Rep' involved in PCV DNA replication. The unique features of PCV, of requiring both Rep and Rep' for replication, complicates analysis based on our understanding of other systems. It remains unresolved why Rep' is needed for PCV replication in mammalian cells when Rep–Rep homo-complexes are available. What function(s) does Rep' provide (not provided by Rep) that is required for the replication of the PCV genome in mammalian cells? In addition, there are several major gaps in our understanding of the PCV DNA replication mechanism. Where is the minus-genome primer located and how is it synthesized? How are the host DNA replication proteins activated or recruited to synthesize the PCV genome? What is the exact mechanism for replication termination? Answers to these questions will no doubt provide insight into the mechanisms of RCR in general.

Here, a theory was proposed that speculates on the identity and generation of the minus-genome primer for PCV. As stated above, the minus-genome primers for several members of the geminiviruses and nanoviruses have been reported; however, the process under which they were synthesized has not been determined. PCV has developed a sophisticated mechanism to reproduce itself and its continued existence depends on its ability to convert from a ss genome to a ds-RF in the host. It is unlikely that such a critical step is left to chance encounter with a minus-genome primer. Therefore, it is logical to expect that synthesis of this primer is an integral part of the replication strategy. The 10–12 nt SP DNA covalently attached to the Rep-complex (SP:Rep-complex) produced at DNA termination is an ideal candidate for being that minus-genome

primer if it segregates together with the ss genome at DNA termination. Although the first two nucleotides at the 5'-end of SP are not complementary to the loop sequence of the plus-strand PCV genome, the remaining 8–10 nt should be able to base-pair with the left-arm palindrome at the Ori and serve as a primer for DNA synthesis. Studies on the Rep protein of parvoviruses, with similarities to PCV Rep, showed that the parvovirus Rep plays a critical role in assembly of viral particles, translocation of the viral genome into the preformed capsid and becomes part of the infectious virus particle (Bleker et al., 2006; Cotmore and Tattersall, 1989; Dubielzig et al., 1999). The translocation of the parvovirus genome by Rep is helicase-mediated and ATP-dependent (King et al., 2001), which, incidentally, are the predicted functions of the P-loop domain present in the PCV Rep protein (Steinfeldt et al., 2007). It is possible that the SP:Rep-complex associated with the ss viral DNA is the signal for PCV DNA packaging to produce infectious virions. When PCV infects a new cell, the SP:Rep-complex is uncoupled by a cellular enzyme capable of dissociating a tyrosine-linkage from the 5' end of a DNA molecule (Bahmed et al., 2010; Nitiss et al., 2006). After removal of the Rep-complex, the two non-complementary nucleotides present at the 5'-end of SP will then be repaired by the replication machinery during minus-genome synthesis to produce a closed circular ds-RF DNA molecule.

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## References

- Bahmed, K., Nitiss, K.C., Nitiss, J.L., 2010. UnTrapping the ends: a new player in overcoming protein linked DNA damage. *Cell Res.* 20 (2), 122–123.
- Beach, N.M., Juhan, N.M., Cordoba, L., Meng, X.J., 2010a. Replacement of the replication factors of porcine circovirus (PCV) type 2 with those of PCV type 1 greatly enhances viral replication *in vitro*. *J. Virol.* 84 (17), 8986–8989.
- Beach, N.M., Ramamoorthy, S., Opriessnig, T., Wu, S.Q., Meng, X.J., 2010b. Novel chimeric porcine circovirus (PCV) with the capsid gene of the emerging PCV2b subtype cloned in the genomic backbone of the non-pathogenic PCV1 is attenuated *in vivo* and induces protective and cross-protective immunity against PCV2b and PCV2a subtypes in pigs. *Vaccine* 29 (2), 221–232.
- Bleker, S., Pawlita, M., Kleinschmidt, J.A., 2006. Impact of capsid conformation and Rep-capsid interactions on adeno-associated virus type 2 genome packaging. *J. Virol.* 80 (2), 810–820.
- Blinkova, O., Victoria, J., Li, Y., Keele, B.F., Sanz, C., Ndjanga, J.B., Peeters, M., Travis, D., Lonsdorf, E.V., Wilson, M.L., Pusey, A.E., Hahn, B.H., Delwart, E.L., 2010. Novel circular DNA viruses in stool samples of wild-living chimpanzees. *J. Gen. Virol.* 91 (Pt 1), 74–86.
- Bratanich, A.C., Blanchetot, A., 2002. PCV2 replicase transcripts in infected porcine kidney (PK15) cells. *Virus Genes* 25 (3), 323–328.
- Chaiyakul, M., Hsu, K., Dardari, R., Marshall, F., Czub, M., 2010. Cytotoxicity of ORF3 proteins from a nonpathogenic and a pathogenic porcine circovirus. *J. Virol.* 84 (21), 11440–11447.
- Cheung, A.K., 2003a. Comparative analysis of the transcriptional patterns of pathogenic and nonpathogenic porcine circoviruses. *Virology* 310 (1), 41–49.
- Cheung, A.K., 2003b. The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. *Virology* 313 (2), 452–459.
- Cheung, A.K., 2003c. Transcriptional analysis of porcine circovirus type 2. *Virology* 305 (1), 168–180.
- Cheung, A.K., 2004a. Detection of template strand switching during initiation and termination of DNA replication of porcine circovirus. *J. Virol.* 78 (8), 4268–4277.
- Cheung, A.K., 2004b. Identification of an octanucleotide motif sequence essential for viral protein, DNA, and progeny virus biosynthesis at the origin of DNA replication of porcine circovirus type 2. *Virology* 324 (1), 28–36.
- Cheung, A.K., 2004c. Identification of the essential and non-essential transcription units for protein synthesis, DNA replication and infectious virus production of Porcine circovirus type 1. *Arch. Virol.* 149 (5), 975–988.
- Cheung, A.K., 2004d. Palindrome regeneration by template strand-switching mechanism at the origin of DNA replication of porcine circovirus via the rolling-circle melting-pot replication model. *J. Virol.* 78 (17), 9016–9029.
- Cheung, A.K., 2005a. Detection of rampant nucleotide reversion at the origin of DNA replication of porcine circovirus type 1. *Virology* 333 (1), 22–30.

- Cheung, A.K., 2005b. Mutational analysis of the direct tandem repeat sequences at the origin of DNA replication of porcine circovirus type 1. *Virology* 339 (2), 192–199.
- Cheung, A.K., 2006. Rolling-circle replication of an animal circovirus genome in a theta-replicating bacterial plasmid in *Escherichia coli*. *J. Virol.* 80 (17), 8686–8694.
- Cheung, A.K., 2007. A stem-loop structure, sequence non-specific, at the origin of DNA replication of porcine circovirus is essential for termination but not for initiation of rolling-circle DNA replication. *Virology* 363 (1), 229–235.
- Cheung, A.K., Greenlee, J.J., 2011. Identification of an amino acid domain encoded by the capsid gene of porcine circovirus type 2 that modulates intracellular viral protein distribution during replication. *Virus Res.* 155 (1), 358–362.
- Choi, I.R., Stenger, D.C., 1995. Strain-specific determinants of beet curly top geminivirus DNA replication. *Virology* 206 (2), 904–912.
- Choi, I.R., Stenger, D.C., 1996. The strain-specific cis-acting element of beet curly top geminivirus DNA replication maps to the directly repeated motif of the ori. *Virology* 226 (1), 122–126.
- Clark, E.G., 1996. Pathology of the post-weaning multisystemic wasting syndrome of pigs. *Proc. West Can. Assoc. Swine Pract.*, 22–25.
- Cotmore, S.F., Tattersall, P., 1989. A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. *J. Virol.* 63 (9), 3902–3911.
- del Solar, G., Giraldo, R., Ruiz-Echevarria, M.J., Espinosa, M., Diaz-Orejas, R., 1998. Replication and control of circular bacterial plasmids. *Microbiol. Mol. Biol. Rev.* 62 (2), 434–464.
- del Solar, G., Moscoso, M., Espinosa, M., 1993. Rolling circle-replicating plasmids from gram-positive and gram-negative bacteria: a wall falls. *Mol. Microbiol.* 8 (5), 789–796.
- Donson, J., Morris-Krsinich, B.A., Mullineaux, P.M., Boulton, M.I., Davies, J.W., 1984. A putative primer for second-strand DNA synthesis of maize streak virus is virion-associated. *EMBO J.* 3 (13), 3069–3073.
- Dubielzig, R., King, J.A., Weger, S., Kern, A., Kleinschmidt, J.A., 1999. Adeno-associated virus type 2 protein interactions: formation of pre-encapsidation complexes. *J. Virol.* 73 (11), 8989–8998.
- Fauquet, C.M., Fargette, D., 2005. International committee on taxonomy of viruses and the 3,142 unassigned species. *Virol. J.* 2, 64.
- Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. *J. Virol.* 78 (12), 6297–6303.
- Finsterbusch, T., Steinfeldt, T., Caliskan, R., Mankertz, A., 2005. Analysis of the sub-cellular localization of the proteins Rep, Rep' and Cap of porcine circovirus type 1. *Virology* 343 (1), 36–46.
- Gibbs, M.J., Weiller, G.F., 1999. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. *Proc. Natl. Acad. Sci. U.S.A.* 96 (14), 8022–8027.
- Graham, F.L., Rudy, J., Brinkley, P., 1989. Infectious circular DNA of human adenovirus type 5: regeneration of viral DNA termini from molecules lacking terminal sequences. *EMBO J.* 8 (7), 2077–2085.
- Gutierrez, C., 1999. Geminivirus DNA replication. *Cell Mol. Life Sci.* 56 (3–4), 313–329.
- Hafner, G.J., Harding, R.M., Dale, J.L., 1997. A DNA primer associated with banana bunchy top virus. *J. Gen. Virol.* 78 (Pt 2), 479–486.
- Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S., Robertson, D., 2000. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Crit. Rev. Biochem. Mol. Biol.* 35 (2), 105–140.
- Harding, J.C., 1996. Post-weaning multisystemic wasting syndrome (PMWS): preliminary epidemiology and clinical presentation. *Proc. West Can. Assoc. Swine Pract.* 21.
- Ilyina, T.V., Koonin, E.V., 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eukaryotes and archaeobacteria. *Nucleic Acids Res.* 20 (13), 3279–3285.
- Jupin, I., Hericourt, F., Benz, B., Gronenborn, B., 1995. DNA replication specificity of TYLCV geminivirus is mediated by the amino-terminal 116 amino acids of the Rep protein. *FEBS Lett.* 362 (2), 116–120.
- Kato, M., Hokabe, S., Itakura, S., Minoshima, S., Lyubchenko, Y.L., Gurkov, T.D., Okawara, H., Nagayama, K., Shimizu, N., 2003. Interarm interaction of DNA cruciform forming at a short inverted repeat sequence. *Biophys. J.* 85 (1), 402–408.
- Kato, M., Matsunaga, K., Shimizu, N., 1998. A novel unusual DNA structure formed in an inverted repeat sequence. *Biochem. Biophys. Res. Commun.* 246 (2), 532–534.
- Khan, S.A., 2000. Plasmid rolling-circle replication: recent developments. *Mol. Microbiol.* 37 (3), 477–484.
- Khan, S.A., Carleton, S.M., Novick, R.P., 1981. Replication of plasmid pT181 DNA in vitro: requirement for a plasmid-encoded product. *Proc. Natl. Acad. Sci. U.S.A.* 78 (8), 4902–4906.
- King, J.A., Dubielzig, R., Grimm, D., Kleinschmidt, J.A., 2001. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. *EMBO J.* 20 (12), 3282–3291.
- Li, L., Kapoor, A., Slikas, B., Bamidele, O.S., Wang, C., Shaukat, S., Masroor, M.A., Wilson, M.L., Ndjanga, J.B., Peeters, M., Gross-Camp, N.D., Muller, M.N., Hahn, B.H., Wolfe, N.D., Triki, H., Bartkus, J., Zaidi, S.Z., Delwart, E., 2010. Multiple diverse circoviruses infect farm animals and are commonly found in human and chimpanzee feces. *J. Virol.* 84 (4), 1674–1682.
- Lin, W.L., Chien, M.S., Du, Y.W., Wu, P.C., Huang, C., 2009. The N-terminus of porcine circovirus type 2 replication protein is required for nuclear localization and ori binding activities. *Biochem. Biophys. Res. Commun.* 379 (4), 1066–1071.
- Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *J. Virol.* 79 (13), 8262–8274.
- Mankertz, A., Hillenbrand, B., 2001. Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. *Virology* 279 (2), 429–438.
- Mankertz, A., Hillenbrand, B., 2002. Analysis of transcription of Porcine circovirus type 1. *J. Gen. Virol.* 83 (Pt 11), 2743–2751.
- Mankertz, A., Mueller, B., Steinfeldt, T., Schmitt, C., Finsterbusch, T., 2003. New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus types 1 and 2. *J. Virol.* 77 (18), 9885–9893.
- Mankertz, A., Persson, F., Mankertz, J., Blaess, G., Buhk, H.J., 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. *J. Virol.* 71 (3), 2562–2566.
- Mankertz, J., Buhk, H.J., Blaess, G., Mankertz, A., 1998. Transcription analysis of porcine circovirus (PCV). *Virus Genes* 16 (3), 267–276.
- Michel, B., Ehrlich, S.D., 1986a. Illegitimate recombination at the replication origin of bacteriophage M13. *Proc. Natl. Acad. Sci. U.S.A.* 83 (10), 3386–3390.
- Michel, B., Ehrlich, S.D., 1986b. Illegitimate recombination occurs between the replication origin of the plasmid pC194 and a progressing replication fork. *EMBO J.* 5 (13), 3691–3696.
- Musatov, S.A., Scully, T.A., Dudus, L., Fisher, K.J., 2000. Induction of circular episomes during rescue and replication of adeno-associated virus in experimental models of virus latency. *Virology* 275 (2), 411–432.
- Niagro, F.D., Forsthoefel, A.N., Lawther, R.P., Kamalanathan, L., Ritchie, B.W., Latimer, K.S., Lukert, P.D., 1998. Beak and feather disease virus and porcine circovirus genomes: intermediates between the geminiviruses and plant circoviruses. *Arch. Virol.* 143 (9), 1723–1744.
- Nitiss, K.C., Malik, M., He, X., White, S.W., Nitiss, J.L., 2006. Tyrosyl-DNA phosphodiesterase (Tdp1) participates in the repair of Top2-mediated DNA damage. *Proc. Natl. Acad. Sci. U.S.A.* 103 (24), 8953–8958.
- Novick, R.P., 1998. Contrasting lifestyles of rolling-circle phages and plasmids. *Trends Biochem. Sci.* 23 (11), 434–438.
- Ohta, T., Nettikadan, S., Tokumasu, F., Ideno, H., Abe, Y., Kuroda, M., Hayashi, H., Takeyasu, K., 1996. Atomic force microscopy proposes a novel model for stem-loop structure that binds a heat shock protein in the *Staphylococcus aureus* HSP70 operon. *Biochem. Biophys. Res. Commun.* 226 (3), 730–734.
- Rasooly, A., Novick, R.P., 1993. Replication-specific inactivation of the pT181 plasmid initiator protein. *Science* 262 (5136), 1048–1050.
- Saunders, K., Lucy, A., Stanley, J., 1992. RNA-primed complementary-sense DNA synthesis of the geminivirus African cassava mosaic virus. *Nucleic Acids Res.* 20 (23), 6311–6315.
- Sauvage, V., Cheval, J., Foulongne, V., Gouilh, M.A., Pariente, K., Manuguerra, J.C., Richardson, J., Dereure, O., Lecuit, M., Burguiere, A., Caro, V., Eloit, M., 2011. Identification of the first human gyrovirus, a virus related to chicken anemia virus. *J. Virol.* 85 (15), 7948–7950.
- Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2001. Rep and Rep' protein of porcine circovirus type 1 bind to the origin of replication in vitro. *Virology* 291 (1), 152–160.
- Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2006. Demonstration of nicking/joining activity at the origin of DNA replication associated with the rep and rep' proteins of porcine circovirus type 1. *J. Virol.* 80 (13), 6225–6234.
- Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2007. Functional analysis of cis- and trans-acting replication factors of porcine circovirus type 1. *J. Virol.* 81 (11), 5696–5704.
- Tischer, I., Miels, W., Wolff, D., Vagt, M., Griem, W., 1986. Studies on epidemiology and pathogenicity of porcine circovirus. *Arch. Virol.* 91 (3–4), 271–276.
- Tischer, I., Peters, D., Rasch, R., Pociuli, S., 1987. Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. *Arch. Virol.* 96 (1–2), 39–57.
- Tischer, I., Rasch, R., Tochtermann, G., 1974. Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. *Zentralbl. Bakteriologie Orig. A* 226 (2), 153–167.